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(54) Title: NON-MAMMALIAN GNRH ANALOGS AND USES THEREOF IN TUMOR CELL GROWTH REGULATION AND CANCER THERAPY

(57) Abstract: Specially designed non-mammalian GnRH analogs resistant to degradation by the tumor tissue enzymes, post-proline peptidases as well as endopeptidases, are disclosed. The GnRH analogs are further defined as analogs of chicken II GnRH, salmon GnRH, or herring GnRH, but can include any non-mammalian GnRH analog with similar amino acid structure. These non-mammalian analogs incorporate D-arginine, D-leucine- D-t-Bu-Serine ore D-Trp or other similar amino acids at position 6 and ethylamide or aza-Gly-amine or similar amides at position 10. These analogs demonstrate preferential binding to tumor cell GnRH receptors that is greater relative to the binding of the mammalian analogs to the tumor cell GnRH receptor. These non-mammalian GnRH analogs may be used in pharmaceutical preparations, and specifically in various treatments as an anti-tumor, anti-proliferation, anti-metastatic and/or apoptotic agent. The non-mammalian GnRH analogs are also provided in pharmaceutical preparations that may be used clinically for tumor regression when used in very low doses and administered in pulsatile fashion.

Title: Non-Mammalian GnRH Analogs and Uses Thereof in Tumor Cell Growth Regulation and Cancer Therapy

BACKGROUND OF THE INVENTION

1. Field of the Invention

Applicants' invention relates to the field of tumor growth regulation. More particularly, Applicants' invention concerns unique non-mammalian peptide hormone analogs of non-mammalian gonadotropin releasing hormone (GnRH) and the method for use of these analogs in the regulation of cell growth, particularly cancer cell growth.

2. Background Information

Gonadotropin-releasing hormone (GnRH) is a hormone known to be produced in the hypothalamus with binding affinity for the pituitary gland. When hypothalamic GnRH binds to the pituitary it causes the pituitary gland to release the gonadotropins (i.e. gonad stimulating hormones) luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Each of these pituitary hormones has a different effect depending on one's sex. One important effect is the production and secretion of the gonadal steroids estrogen and progestogen in sexually mature females and testosterone in males.

Before the chemical characterization of the mammalian GnRH it was realized that a hypothalamic substance regulated the production of pituitary LH and FSH (1) and that these gonadotropins regulated gonadal steroidogenesis. The delineation of mammalian GnRH enabled Applicants to synthesize this decapeptide and administer it systemically to humans (2). It was then recognized that a long acting superagonist of this mammalian GnRH effected a flare release of pituitary gonadotropins followed by their inhibition (3). The inhibition was effected by a down-regulation of the pituitary GnRH receptor which corresponded downstream to an inhibition of the gonadal steroids, estrogen, progesterone and/or testosterone.

This is a form of chemical castration.

Since certain types of tumors, such as certain breast (4) and prostate cancers (5), are now known to be dependent on gonadal steroids, mammalian GnRH analogs have been used to suppress gonadal steroids via their chemical castration activity (3). Thus, we know that the use of mammalian GnRH analogs is feasible as a treatment of certain cancers.

It was only with the development of sensitive and specific radioimmunoassays for GnRH and GnRH-like molecules that a very surprising finding was reported. That finding was initially described by Applicants. Applicants reported that GnRH-like molecules exist and function not only in the hypothalamic-pituitary axis, which functions as an endocrine system to distribute hormone systemically, but GnRH-like molecules also exist in extra-hypothalamic tissues(6-9) to provide a paracrine action i.e. localized signal secretion. It is now realized that paracrine action of GnRH-like substances have functions in the placenta, gonad, breast, prostate, etc., (10-13) as well as in many cancerous tumors (14-31). Even with this general knowledge, the effective use of mammalian GnRH analogs to act directly on particularly tumor tissue has not resulted. One of the goals of the present invention was to utilize novel forms of GnRH not previously envisioned for cancer therapy that bind to the tumor GnRH-like receptor with 50 to 1000 fold the activity of mammalian GnRH or its superagonist and have potent bioactivity in inhibiting tumor cell growth.

The initial studies on GnRH activity in tumor tissues and the human placenta utilized mammalian GnRH and its analogs, in accordance with the teaching that the human encodes for only one isoform of GnRH (32,33). In Applicants' studies in the human placenta Applicants localized and quantified the concentration of GnRH produced by the human placenta throughout pregnancy (34,35). Applicants demonstrated the synthesis and activity of a GnRH-like molecule in regulating human chorionic gonadotropin (hCG) and steroid production (36-38), and the release of a GnRH-like molecule into the maternal circulation (34). Applicants also demonstrated that high doses of mammalian GnRH could stimulate hCG and

prostanoid production in a specific receptor mediated event and that GnRH-like production and activity in the human placenta is regulated by feedback interactions of estrogens and progesterone production (39-41). Thus, Applicants described and established the first paracrine system in human or mammalian physiology (42-45). Concomitantly, Dubois et al. (12) described a second paracrine system from the presence of somatostatin in the pancreas. Thereafter, Applicants and other investigators reported actions of mammalian GnRH on placental function and identified feedback interactions including activin, inhibitin, follistatin, neurotransmitters, prostaglandins, and steroids (46-63).

Using in situ localization a message to mammalian GnRH has been localized at the syncytio- and cytotrophoblast, as well as the stroma of the placenta (64-66). A gene for mammalian GnRH differing from hypothalamic GnRH by the inclusion of the first intron and a very long first exon has been reported (67-69). Multiple transcription sites have been identified for the GnRH gene in the placenta as well as in other reproductive tissues (70-72). Steroid regulatory sites on the promoter have also been identified (73,74). The functionality of the promoter is supported by the demonstration that mRNA for GnRH can be regulated by steroids (75-78).

Placental GnRH receptor activity and a GnRH mRNA have also been identified (79,80). The receptor number is highest in early gestation and down-regulated by 12-20 weeks, and still detectable in term placenta, although the mRNA for mammalian GnRH is not (79,80). This pattern of receptor activity parallels that of chorionic GnRH-like activity (6,34)and supports the hypothesis that chorionic GnRH may down-regulate its receptor, as does mammalian GnRH and its analogs at the pituitary level. Studies of Szilagyi et al. (81) and Currie et al. (82) have indicated down-regulation of chorionic receptors by mammalian GnRH analogs. In addition, estradiol has been shown to upregulate the placental GnRH receptor. Thus, there is substantial data to indicate a functional, regulated GnRH receptor in extrahypothalamic tissues.

In total, these studies have firmly established the presence of a hypothalamic-pituitary-gonadal axis in extra-hypothalamic tissue. Presently many

other hypothalamic-like activities, such as by corticotropin-releasing hormone (CRH), have now been defined in the placenta and other tissues as well. Such paracrine axis are known in the pancreas, thyroid, gut, bone, brain, ovary, endometrium, eye, etc.

Of particular interest to this invention are previous reports of the presence of GnRH-like substances and receptors in numerous cancer tissues and their cell lines (15,17,20,23,25,26,30,31,83,84). GnRH-like activity and its receptors have been identified in the breast, bronchial, ovarian, endometrial, prostate, gastrointestinal tumors. The function of a GnRH-like substance and its receptors in tumor tissues is supported by the demonstration that mammalian GnRH can stimulate hCG from human and animal tumors and can inhibit cell growth in vitro. These findings have led to numerous studies of the effects of mammalian GnRH analogs on the expression of GnRH receptors, cell signal transduction, apoptosis, and overall growth of tumor cell lines (14,16,18,19,21,22,29,85-89). The growth of tumors in vivo has also been studied with individual case reports of patients responsive to mammalian GnRH analogs (24,27,28,90-92).

However, some very problematic findings from previous studies in both the placenta and tumor tissue has led to skepticism about the true role of mammalian GnRH analogs in both tissues. The GnRH receptor affinity for GnRH in both the placenta and tumors is on the order of 10^{-5} to 10^{-6} M (84,93,94). The biological significance of such a weak affinity in light of much lower levels of endogenous GnRH-like activity must be questioned. In addition, Applicants have observed in human pregnancy studies, both in vitro and in vivo, that mammalian GnRH appears to act as a partial agonist not a true agonist of tumor GnRH(38,95). When receptors are available, mammalian GnRH acts as an agonist of tumor GnRH, but when tumor receptors are low or occupied, mammalian GnRH competes with the more potent tumor GnRH resulting in a partial agonist action. Furthermore, Applicants and others have observed that certain antibodies for mammalian GnRH reacted with chorionic GnRH with a different affinity (96-99). These findings led Applicants to propose that neither the extra-hypothalamic GnRH nor its receptor are

identical to mammalian GnRH and its pituitary receptor (100,101).

Applicants have defined yet another difference in extra-hypothalamic GnRH, i.e., its metabolism. The metabolism of a hormone is as important for maintaining biologically active concentrations of that hormone, as that which stimulates the hormone's synthesis and release. For GnRH, in the non-pregnant human, both in the pituitary and in the circulation, the predominant enzymatic degradation is directed to the 5-6 peptide bond catalyzed by an endopeptidase. Thus, existing analogs of the mammalian GnRH each bear a D-amino acid substituted in the 6 position. However, Applicants have isolated and characterized the dominant enzyme that degrades GnRH in the placenta and it is a post-proline peptidase acting to cleave the proline-glycine peptide bond at the 9-10 position (102,103). Applicants have recently obtained similar data for the metabolism of GnRH in breast tumor cells. Thus, there appears to be cell specific metabolism of GnRH at the placenta and breast tumor cells which differs from that in blood and the pituitary.

Since it appeared as though there was a different form of GnRH at work at the placenta and breast tumor cells, various isoforms of GnRH were investigated. Different isoforms of GnRH have been identified in non-mammalian species, such as fish and aves. The unique sequence of these GnRH are known. Chicken I, chicken II, salmon, catfish, dogfish, lamprey and more recently herring GnRH have also been reported (33,106). In lower vertebrates a number of GnRH isoforms can be expressed in the same species (32,33,76,78,105,107-116). In most cases, each decapeptide conserves the first four, the sixth and in every case, the last two amino acids in the GnRH molecule, but have varying amino acids in the fifth, seventh and/or eighth position. These modifications render the molecule unique, having only weak affinity for the mammalian pituitary receptor, although conversely mammalian GnRH is active in many lower vertebrate classes.

As mentioned, in certain lower vertebrates a number of GnRH isoforms are expressed in the same species. In amphibians, a chicken II GnRH receptor as well as a mammalian GnRH receptor has been reported. However, it was not until 1994, when Dellovade et al. (117) and King et al. (118) described chicken II GnRH in

musk shew, mole and bat brain, that the existence of multiple isoforms of GnRH in a mammal was realized. Even then, it was still thought that modern placental mammalian species did not encode or express other than mammalian GnRH. Recently however, chicken II GnRH has been characterized in the tree shew (119), guinea pig, and primate brain (120) and their separate genes have been described (121,122). Only this year has the code for the chicken II GnRH receptor been identified in human tissues.

In contrast, Applicants have proposed and obtained substantial data to support the hypothesis that non-mammalian isoforms of GnRH and their specific receptors are expressed in extra-hypothalamic tissues and that the non-mammalian GnRH molecules are the true ligands for these receptors (123,124). Applicants have also proposed that these GnRH molecules have specific roles in regulating cell growth and cell death and are pivotal in regulating cell growth of GnRH responsive tumors by a direct receptor mediated action on these tumor cells.

It is believed that the non-mammalian GnRH isoforms and analogs of the present invention may act either as a superagonist at the tumor tissue leading to tissue receptor down-regulation, or as a pure antagonist of the endogenous isoform of GnRH in the tumor tissue, acting via the tumor tissue receptor. The down-regulation of the GnRH receptor or the antagonism of the endogenous isoform of GnRH will provide for a reduction in cell proliferation and/or induce apoptosis. The specific action of the non-mammalian GnRH analog will compete at the tumor cell GnRH receptor(s) with the endogenous isoform of GnRH effecting an antagonism or a superagonistic down-regulation of the receptor, leading to cell death and regression of the tumor and inhibition of metastasis. Thus, this agent may be used to reduce tumor growth. To date, no such non-mammalian GnRH analog has been designed which has stability and tumor tissue specificity.

To date, little if any data, has been reported in relation to non-mammalian GnRH activity on tumor tissues. Chicken I GnRH and Lamprey GnRH (18,86,125,126) have been studied and limited activity was found. Applicants have studied these isoforms of GnRH and have found no or limited binding activity

in chorionic tissues. On the other hand, Applicants have demonstrated greatly enhanced binding and bioactivity of chicken II GnRH and salmon GnRH analogs as compared to mammalian GnRH or its analogs in both breast cancer cells and placental tissue. Thus, Applicants have obtained data to support the hypothesis that certain non-mammalian GnRH analogs have enhanced receptor and bioactivity for tumor tissues and this finding taken together with the understanding of the unique metabolism of GnRH isoforms in cell specific sites have formed the basis of Applicants invention, i.e., the utilization of stable, cell-active analogs of non-mammalian GnRH isoforms to regulate tumor cell growth and the treatment of cancer. In addition, Applicants postulate that due to similar amino acid structures, Herring GnRH, Dogfish GnRH, and Catfish GnRH as well as other GnRH isoforms and analogs with similar amino acid structure should exhibit the same or similar binding and bioactivity.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel pharmaceutical preparation that includes non-mammalian GnRH isoforms and their analogs specifically designed to bind to extra-pituitary GnRH receptors expressed on tumor tissues.

It is another object of the present invention to provide novel GnRH analogs that are resistant to degradation by post-proline peptidases, particularly those which are known to be found around tumor tissues.

Still another object of the present invention is to provide novel GnRH analogs that are resistant to the endopeptidase found circulating in the blood.

Another object of the present invention is to provide novel GnRH analogs that act as superagonists at the tumor tissue leading to tissue receptor down regulation.

Yet another object of the present invention is to provide novel GnRH analogs that act as pure antagonists of the endogenous form of GnRH in tumor tissue via the tumor tissue receptor.

Another object of the present invention is to provide novel GnRH analogs

which reduce tumor cell proliferation.

Still another object of the present invention is to provide novel GnRH analogs which induce apoptosis.

It is yet another object of the present invention to provide novel GnRH analogs which reduce tumor cell metastasis.

Another object of the present invention is to provide novel GnRH analogs that can be used as anti-tumor agents.

Yet another object of the present invention is to provide novel GnRH analogs that can be used to reduce tumor cell growth.

It is still another object of the present invention to provide novel GnRH analogs that are stable in circulation.

Another object of the present invention is to provide novel GnRH analogs that are tumor tissue specific.

Yet another object of the present invention is to provide novel GnRH analogs which can be used to induce tumor regression.

Still another object of the present invention is to provide a novel method for synthesizing analogs of non-mammalian GnRH isoforms having increased activity in tumor tissues.

It is another object of the present invention to provide a novel method for inhibiting tumor growth which in turn reduces tumor cell proliferation, tumor size and metastasis i.e. apoptosis and tumor regression.

It is yet another object of the present invention to provide a novel method for using non-mammalian GnRH analogs directly on tumors as an anti-tumor or anti-metastasis drug.

Another object of the present invention is to provide a novel non-mammalian GnRH analog composed of Salmon, Chicken II, or Herring GnRH analogs that are modified at the C-terminal and which shown greater affinity for the tumor receptor than mammalian GnRH.

It is still another object of the present invention to provide a novel non-mammalian GnRH analog which has an aza-Gly-NH_2 substitution at the number 10

position to make the sequence more stable in tumor tissues and in blood and to inhibit degradation by post-proline peptidases.

Yet another object of the present invention is to provide a novel non-mammalian GnRH analog sequence which is substituted at the 6 position with preferably a D-Arg but could be any other D-amino acid such as, but not limited to, D-Leu, D-Trp, and D-Bu-Ser.

It is another object of the present invention to provide a novel non-mammalian GnRH analog which has increased binding affinity to the tumor receptor and metabolic stability.

Still another object of the present invention is to provide a novel non-mammalian GnRH analog which will not be toxic after long term therapies.

Yet another object of the present invention is to provide a novel more potent non-mammalian GnRH analog which can be used to bind to the tumor tissue GnRH receptor with high affinity so as to displace the endogenous GnRH-like activity and block its action.

It is another object of the present invention to provide a novel non-mammalian GnRH analog which incorporates a substitution of Gly(10)-NH_2 with ethylamide to inhibit degradation by post-proline peptidases.

Still another object of the present invention is to provide a novel non-mammalian GnRH analog which has minimal effect on the mammalian pituitary GnRH receptor.

In satisfaction of these and related objectives, Applicant's present invention provides unique non-mammalian peptide hormone analogs of non-mammalian GnRH and the method for use of these analogs in the regulation of cell growth, particularly tumor cell growth. Applicant's invention permits its practitioner to treat patients who have cancerous tumors with non-mammalian GnRH analogs which have high binding affinity to the GnRH receptors located on tumor cells which in turn reduces the tumor size and proliferation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Chart of the sequences of known isoforms of GnRH.

Figure 2. Effect of des-Gly(10)-mammalian GnRH-ethylamide on the degradation of mammalian GnRH by the chorionic post-proline peptidase.

◊ GnRH 0.00313 M, ∇ GNRH 0.0625 M, ●GNRH 0.0125 M.

Figure 3. Action of D-Arg- Chicken II-aza-Gly-NH_2 on the Degradation of Mammalian GnRH by Chorionic Post-Proline Peptidase. ◊ GnRH 0.00313 M, ∇ GNRH 0.0625 M, ● GNRH 0.0125 M, 0 GNRH 0.0250 M.

Figure 4. Binding of I^{125}-D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide to MCF 7 breast cancer cells after 24 hours of incubation with no exogenous GnRH or competing isoforms or analogs of GnRH

Figure 5. The anti proliferative, tumor regression activity of D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide compared to controls and other isoforms and analogs of GnRH after 24 hours.

Figure 6. Inhibitor constants for analogs of GnRH.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Following long-standing patent law convention, the terms 'a" and "an" mean "one or more" when used in this application, including the claims.

It should be appreciated by those of skilled in the art that the techniques disclosed in the material which follows represent techniques discovered by the Applicants to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those skilled in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Design & Synthesis of Non-Mammalian GnRH Analogs

The preferred embodiment for the present invention is the design of non-mammalian GnRH analogs that have increased activity in tumor tissue cells by exhibiting potent tumor receptor binding activity, tumor tissue stability, and biological activity.

Existing mammalian GnRH analogs are designed for activity at the pituitary GnRH receptor and with extended stability in the circulation of non-pregnant individuals i.e. protection from endopeptidase degradation. Yet, existing data indicate that the tumor GnRH receptor differs from that in the pituitary. Therefore, prior known pituitary mammalian GNRH analogs have not been designed for direct use in tumor tissues and potent non-mammalian GnRH analogs have not been designed for use to regulate cancer cell growth. The present invention provides potent non-mammalian GnRH analogs that act directly on tumor cell growth and proliferation.

Non-mammalian analogs of GnRH were designed according to general guidelines established by Applicants. First, these analogs were specifically designed to be resistant to degradation both in the maternal circulation as well as within the tumor tissue by endopeptidases and post-proline peptidases. This allows for the maintenance of sufficient concentrations of analog when administered via the maternal system to reach the cancerous tissue. And second, the analogs were designed according to the particular specificity of the tumor cell receptor to specifically bind the GnRH receptors with high affinity so as to preferably displace the endogenous GnRH activity and block its action. This tumor GnRH binding specificity can effect either a down regulation of the tumor GnRH receptor or act as a true antagonist to inhibit tumor growth, proliferation, and metastasis by inducing apoptosis and tumor regression by directly inhibiting tumor function.

Analogs of the Salmon and Chicken II GnRH sequences, that show greater affinity for the tumor GnRH receptor than for the pituitary GnRH receptor, were modified to the aza-Gly(10)-NH_2 analog to make them resistant to degradation by post-proline peptidases (See Figure 1, Analogs 2, 4 and 6). Ethylamide or other similar amides can be used at position 10. Next, the Chicken II GnRH sequence, and the Salmon GnRH sequence were modified at the 6 position using D-Arg, D-Leu, D-tBu-Ser, D-Trp, or other similar amino acid making the analog resistant to degradation by the endopeptidase in blood, and at the 10 position using preferably aza-Gly(10)-NH_2, making it stable in maternal blood and the tumor tissues (See

Figure 1, Analogs 2, 4 and 6). Again, ethylamide or other similar amides can be used. These analogs are expected to have not only increased binding to the tumor receptor but also increased metabolic stability. Due to the similarity in amino acid makeup, these procedures could be repeated using Herring GnRH, Dogfish GnRH, and Catfish GnRH as well as any other decapeptide with similar amino acid structure. The preparation and chemical manipulation of these non-mammalian GnRH analogs can be completed with any standard protocol.

Tumor Receptor Binding of GnRH Isoforms and Analogs

The tumor receptor binding activity of the different non-mammalian GnRH analogs of the present invention was compared. Prior mammalian GnRH analogs have been designed to increase activity at the pituitary GnRH receptor and stability in the circulation of nonpregnant individuals. These analogs do not demonstrate as potent binding activity at the tumor receptor as they do at the pituitary receptor. In contrast and as was mentioned earlier, the non-mammalian GnRH analogs of the present invention have been designed to interact with preference at the tumor receptor and not the pituitary receptor.

GnRH receptors on the cells of MCF-7 breast cancer cells were studied. The cells were plated on 96 wells and grown to confluency in base medium (M3D:Fetal Bovine Serum [9:1]). Prior to the experiment the cells were down shifted to M3D:Fetal Bovine Serum [99:1] and then to serum-free medium. Cells were incubated for 24 hours at room temperature with mammalian GnRH, Buserilin, Leuprolide, Chicken II GnRH, and D-Arg(6) Chicken II-aza-Gly(10)-amide. Cells were then collected and studied for receptor binding and receptor number with D-Arg(6) Chicken II-aza-Gly(10)-amide. Addition of enzyme inhibitors of the endogenous post-proline peptidase and other peptidases were used as well as agents for receptor stabilization. Receptor bound label was separated by centrifugation. The binding of the non-mammalian GnRH analog and its ability to regulate the tumor cells' GnRH receptor was compared. Figure 4 shows the binding of I^{125} -D-Arg(6)-Chicken II GnRH-aza-Gly (10) -amide to MCF-7 breast cancer cells after 24 hours of incubation with no exogenous GnRH or competing

isoforms or analogs of GnRH. D-Arg(6) -Chicken II GnRH-aza-Gly(10)-amide specifically bound the MCF-7 breast cancer cells. This binding was competitively inhibited by D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide with the greatest potency. This was followed by Buserilin. Mammalian GnRH was the weakest competitor, while Chicken II GnRH was highly potent even though it is not protected from degradation either at the 6 or the 10 position. This indicated the high innate affinity of this isoform of GnRH for the tumor GnRH receptor. It is believed by Applicants that the same or similar results could be obtained using other non-mammalian GnRH isomers or analogs with similar amino acid structure as Chicken II GnRH such as but not limited to Salmon GnRH, Herring GnRH, Catfish GnRH, or Dogfish GnRH.

Tumor Tissue Stability Studies for GnRH Isoforms and Analogs

As has been previously mentioned, mammalian GnRH and its analogs bind with weak affinity to tumor cell receptors in certain tumor tissues whereas the non-mammalian GnRH analogs exhibit strong affinity for these receptors. Observing this strong affinity it became necessary to examine the non-mammalian GnRH analogs for stability. The non-mammalian GnRH analogs of the present invention have not previously been examined for stability. However, the added stability of these non-mammalian analogs would effect a substantial increase in bioactivity. Thus, stability studies involving endopeptidase and post-proline peptidase were performed for the non-mammalian GnRH analogs.

Endopeptidase Stability Studies:

Since human pituitary and blood contain an enzymatic activity that degrades GnRH at the 5-6 position, rather than the 9 position, present non-mammalian GnRH analogs have been designed to inhibit the former enzymatic activities and have substitutions in the 5-6 position of the molecule. The present analogs with these substitutions are therefore resistant to degradation at the pituitary or in the blood of non-pregnant individuals. However, these substitutions alone do not protect the analogs from degradation at the tumor tissues which contain post-proline peptidase. Substitution of the Gly(10)-NH_2 with ethylamide, or the more potent

aza-Gly(10)-NH_2, inhibits degradation by post-proline peptidases. A number of existing mammalian GnRH analogs also have a substitution of Gly(10)-NH_2.

Post-Proline Peptidase Stability Studies:

As mentioned earlier, the post-proline peptidase is important in actively degrading peptides that contain a proline residue. GnRH is such a peptide. Initially the enzymatic activity of the tumor cell was studied. Tumor tissue cells and their spent media were studied for enzyme activity. In particular, examination was made for the degradation of GnRH both with and without specific post-proline and endopeptidase activity inhibitors to determine the specificity of the tumor enzymatic activity. These studies have demonstrated very high post-proline peptidase activity produced by the tumor tissue.

The enzymatic degradation of the non-mammalian GnRH analogs were studied in MCF-7 breast cancer cells using an enzymatic activity assay and compared to that for the purified chorionic post-proline peptidase. Chorionic post-proline peptidase is a peptidase with high specificity for the degradation of GnRH at the proline-glycine bond, but can also degrade other GnRH species containing this bond.

In a non-pregnant individual very little post-proline peptidase activity is present in the blood or the pituitary. Thus, currently available mammalian GnRH analogs have not been designed to be resistant to degradation by this activity. However, due to the high post-proline peptidase activity present in tumor tissue, the non-mammalian GnRH analogs for cancer therapy described herein were designed to be resistant to this type of degradation. The stability of these non-mammalian GnRH analogs in the presence of post-proline homogenates was examined and compared to existing mammalian GnRH analogs. In addition, the ability of the analogs to competitively inhibit the degradation of GnRH using chorionic post-proline peptidase was studied.

The stability of most potent receptor-active non-mammalian GnRH analogs in the presence of tumor tissue cells, spent media, or tumor tissue cells homogenates was identified. Using the incubation system developed for chorionic post-proline

peptidase activity, the degradation of GnRH was tested. Each of these analogs were first studied for their ability to act as a competitive inhibitor of GnRH for chorionic post-proline peptidase activity using the enzymatic activity assay as described previously (103). In this assay, incubation of enzyme and mammalian GnRH with and without the chosen newly synthesized non-mammalian GnRH analog was studied. The reaction was stopped by heating at 85°C for 10 minutes. The remaining mammalian GnRH substrate was quantified by radioimmunoassay. The product formed, i.e. the N-terminal nonapeptide of GnRH, was calculated by subtraction, and its inverse plotted against the inverse of the original substrate concentrations to determine the Ks of the competition. The Ki was determined by plotting the inverse of the product that formed versus the inhibitor used. The inhibitory activity of Antide, Im-btl-D-His(6)-mammalian-GnRH-ethylamide, D-Trp(6)-GnRH-ethylamide, Buserilin, Leuprolide, OH-Pro(9)-Mammalian GnRH, Mammalian GnRH-ethylamide, Chicken II GnRH, Chicken II-ethylamide, D-Arg(6)-Chicken II-ethylamide, D-Arg(6)-Chicken II-aza-Gly(10)-amide, Chicken I GnRH, Salmon GnRH, D-Arg(6)-Salmon GnRH-aza-Gly(10)-amide, and Lamprey GnRH was studied.

Mammalian GnRH was actively degraded by chorionic post-proline peptidase. While replacement of Gly(10)-NH_2 with ethylamide made each of the mammalian GnRH analogs more resistant to degradation than Mammalian GnRH alone, some of these Mammalian GnRH were still degraded by the post-proline peptidase. Of four mammalian GnRH ethylamides studied, des-Gly(10)-GnRH ethylamide, des-Gly(10), D-Trp(6)-GnRH ethylamide, des-Gly(10)-D-Leu(6)-GnRH ethylamide, and Buserilin, each competitively inhibited the degradation of mammalian GnRH; thus they were degraded by the post-proline peptidase. The effect of des-Gly(10) GnRH on the degradation of mammalian GnRH by chorionic post-proline peptidase is shown in Figure 2. The less an analog is capable of competing with the GnRH for the post-proline peptidase, the more resistant it is to degradation by post-proline peptidase and the more stable the analog will be in the tumor tissue and/or in the blood. Thus the existing mammalian GnRH analogs

commonly used in medicine can be degraded in tumor tissues.

This activity of chorionic post-proline peptidase was inhibited by OH-Pro(9)-GnRH, Lamprey GnRH, Chicken I GnRH, Antide, Chicken II GnRH, and Salmon GnRH with a relative potency of 1.5, 1.5, 0.6, 0.6, 0.2, and 0.2, respectively, compared to that for GnRH. In viewing this data, the OH-Pro(9)-GnRH and Lamprey GnRH were determined to be the best competitors for GnRH degradation by chorionic post-proline peptidase. (See Figure 6 for inhibitor constants for analogs of GnRH.) They are as or even more potent than mammalian GnRH. Antide and Chicken I GnRH are three fold less potent than GnRH, but two fold more potent than the Salmon GnRH or Chicken II GnRH. The addition of the ethylamide to mammalian GnRH, both with and without the D-Trp(6)-, D-Phe(6) substitution, decreased the competition with mammalian GnRH for chorionic post-proline peptidase degradation, but not as markedly as did the Im-btl-D-His(6) or Chicken II GnRH analogs. Both D-Arg(6)-Chicken II GnRH-aza-Gly(10)- amide and Im-btl-D-His(6)-GnRH-ethylamide were essentially inactive, i.e. <0.005 inhibitory activity for GnRH. Essentially these latter two GnRH's were greater than 200 fold less active in the inhibition of GnRH degradation by chorionic post-proline peptidase. Thus these analogs appear to be very stable in the presence of post proline peptidase activity, however the Im-btl-His(6) analog has reduced receptor potency. The stability of the D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide was found to not only be greater than 200 fold more stable than GnRH but it still has increased receptor potency. The action of D-Arg(6)-Chicken II GnRH-aza-Gly(10)- amide on the degradation of mammalian GnRH by chorionic post-proline peptidase is shown in Figure 3. It is believed by Applicants that the same or similar results could be obtained using non-mammalian GnRH isomers or analogs with similar amino acid structure as Chicken II GnRH such as but not limited to Herring GnRH, Dogfish GnRH, or Catfish GnRH.

Since chorionic post-proline peptidase is a peptidase with high specificity for the degradation of GnRH at the proline-glycine peptide bond it can also degrade other GnRH species containing the same bond. The synthetic mammalian GnRH

analogs such as Antide are degraded with reduced activity while other analogs such as D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide are resistant to degradation by this endogenous chorionic enzyme. Such a resistant analog can be useful in the regulation of tumor tissue GnRH activity.

Degradation of mammalian GnRH by the tumor tissue cells was essentially 100% after overnight incubation. Specific inhibitors of post-proline peptidase were used to demonstrate this activity in the tumor cell extracts. The degradation of mammalian GnRH was inhibited by bacitracin, but not EDTA, demonstrating the enzyme similarity to chorionic post proline peptidase. From this study it was found that the aza-Gly(10)amide derivatives of Chicken II GnRH and Salmon GnRH have little if any degradation as compared to mammalian GnRH. Each Chicken II and its analogs were more stable than the mammalian GnRH analogs analyzed. It is believed by Applicants that the same or similar results could be obtained using non-mammalian GnRH isomers or analogs with similar amino acid structure as Chicken II GnRH such as but not limited to Herring GnRH, Dogfish GnRH, or Catfish GnRH.

Although the enzyme competition system had already been developed, newly synthesized non-mammalian GnRH analogs have not been utilized in this system. Previous data generated by Applicants have demonstrated that the antiserum is specific for mammalian GnRH, thus reducing potential for cross-reaction of non-mammalian GnRH isoforms or its analogs in the assay used in these studies.

Biological Activity Studies

The tumor growth inhibiting activity of the non-mammalian GnRH analogs was studied. Such data can be used to determine biological activity including regulation of tumor cell growth, tumor proliferation, and tumor regression. Bio-potency was studied by determining cell death and tumor regression. Thus a primary parameter of interest was indicating the cell viability in the tumor cells being regulated by the exogenous GnRH activities which were studied.

The biological activity of the newly synthesized non-mammalian GnRH analogs was studied using an in vitro human tumor cell culture system. This system allows for replicated extended activity studies. Mammalian GnRH action on the tumor

tissue cell has been studied using a similar system. Applicants studied replicate cultures, thus allowing for comparison of different doses of each non-mammalian GnRH analog to mammalian GnRH. In these studies, the action of the most stable and receptor-active non-mammalian GnRH analogs on tumor cell viability were determined.

The bio-potency studies were done with a MCF-7 breast cancer cell culture system and the cell viability as a measure of survival was assessed using the Alamar Blue assay. The percent difference in the Alamar Blue optical density (OD) readings at 570 and 600 nm in the treated and untreated controls was determined. These studies were done using mammalian GnRH, chicken II GnRH, Leuprolide, Buserelin, the D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide analog as well as the D-Leu(6)-Chicken II GnRH-aza-Gly(10)-amide analog. A dose-response study in quadruplicate cultures was performed. Cell viability was assessed after 24 and 48 hours of incubation with the activity agent. The data analysis of these tumor cell culture sets at 24 hours is shown in Figure 5. More specifically, Figure 5 illustrates the anti-proliferative , tumor regression activity of D-Arg(6)-Chicken II GnRH -aza-Gly(10)-amide as compared to controls and other isoforms and analogs of GnRH after 24 hours of incubation. In this Figure 5, A1 is medium 199 (no vehicle); A2 is medium 199 (with vehicle); B1-B3 is Leuprolide; C1-C3 is mammalian GnRH; D1-D3 is Chicken II GnRH; E1-E3 is D-Arg(6)-Chicken II GnRH-aza-Gly(10) amide; G1-G3 is Buserelin.

After 24 hours of incubation, an inhibition of cell proliferation was observed with the Chicken II GnRH and its analogs, while even the highest doses of mammalian GnRH analogs, Leuprolide, and Buserelin were totally inactive. (See Figure 5). The lowest dose of Chicken II studied (10^{-8} M) was more effective than 10^{-5} M mammalian GnRH. The D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide resulted in positive dose-related activity, which was markedly active at 10^{-5} M. After 48 hours of incubation this analog was equally as potent as at 24 hours, while its natural isoform lost potency due to degradation. The mammalian GnRH and its analogs were totally ineffective in the inhibition of the MCF-7 breast cancer cell

proliferation after 48 hours of continued exposure. These data demonstrate that D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide analog is a very stable and bioactive molecule in the regulation of tumor cell growth in the human MCF-7 breast cancer cells. It is believed by Applicants that the same or similar results could be obtained using non-mammalian GnRH isomers or analogs with similar amino acid structure to Chicken II GnRH such as but not limited to Salmon GnRH, Herring GnRH, Dogfish GnRH, or Catfish GnRH.

Using an in vitro system to define bio-potency is expected to be predictive of in vivo activity. In addition to tumor cell action, since these newly synthesized non-mammalian GnRH analogs are known to act directly at the placenta to inhibit steroidogenesis, these analogs are expected to be active at the ovarian level to inhibit steroidogenesis. This would act as an added benefit in the cancer therapy.

Methods for Regulating Tumor Cell Growth and Proliferation In Vivo

In vivo trials utilizing the non-mammalian GnRH analogs of the present invention may be performed to inhibit tumor cell growth and proliferation to thus induce regression of cancer cells in a mammal. The mammal can include a human with cancer. As a proposed dose regimen, it is anticipated that a human between 100 lbs. and 250 lbs. be administered about 10 nanograms to 1.0 gram of a chicken II GnRH analog, salmon GnRH analog, or other non-mammalian GnRH analog with similar amino acid structure. This would be expected to be effective for inhibiting tumor growth or metastasis in the mammal once administered.

It is envisioned that these non-mammalian GnRH analogs will be administered intra-nasally, orally, intramuscularly, transdermally or vaginally. However, virtually any mode of administration may be used in the practice of the invention. Treatment with these analogs may require short-term, repeated administrations of the active non-mammalian GnRH analog or long-term continuous therapy until tumor regression has occurred. Repeated administration could be used as needed.

Numerous in vitro fertilization (IVF) protocols now routinely use mammalian GnRH analogs for ovulation timing and have been shown to be nontoxic, even after weeks of administration. Long-term therapies with mammalian GnRH analogs have

been used for treatment of endometrious, prostate cancer and other cancers and have been shown to be nontoxic, even after months of administration. Long-term therapies with mammalian GnRH analogs have been associated with a hypoestrogenic state, which is frequently a desired condition in certain cancer therapies. The effect on the pituitary GnRH receptor is expected to be minimal with these non-mammalian GnRH analogs and with this short duration of treatment. Thus, the specific receptor activity of these analogs makes it less likely to interfere with normal physiology.

In some trials, the dosing regimen can comprise a pulsatile administration of the analog over a 24-hour period, wherein the daily dosage is administered in relatively equal 1/24th fractions. For example, where the daily dose is about 2.4 micrograms, the patient would be administered about 0.1 micrograms per hour over a 24-hour period. Such a daily pulsatile administration would create a hormonal environment in the patient sufficient to inhibit tumor cell growth and proliferation and/or induce its regression. The particular pharmaceutical preparations may be created by one of skill in the pharmaceutical arts. Remington's Pharmaceutical Sciences Remington: The Science and Practice of Pharmacy, 19" edition, Vol. 102, A. R. Gennaro, ed., Mack Publishing co. Easton, PA. (1995), is specifically incorporated herein by reference for this purpose.

ANTIBODIES SPECIFIC FOR NON-MAMMALIAN GNRH

Another embodiment of the present invention is to utilize non-mammalian GnRH analogs to prepare antibodies that preferentially bind the non-mammalian GnRH peptide sequences, or that bind the tumor tissues or any other non-pituitary GnRH peptide or protein. It is anticipated that these non-mammalian GnRH antibodies may be used in a variety of screening assays. For example, these antibodies may be used to determine levels of GnRH are present in a sample as an indicator molecule. The levels of such GnRH may be used to monitor and follow a patient's tumor activity or growth, as well as an indicator of the tumor's presence. The antibodies to non-mammalian GnRH may be monoclonal or polyclonal antibodies.

Polyclonal antibodies may be created by standard immunization techniques, wherein the immunogen used will be the non-mammalian chicken II GnRH, salmon GnRH, herring GnRH analog, or the naturally occurring decapeptide of any of these described herein, or any other non-mammalian GnRH analog with similar amino acid structure. These peptides may be used either alone or together in a pharmaceutically acceptable adjuvant. The animal, such as a rabbit, would be administered several doses of the decapeptide preparation, and the levels of the animal's antibody blood levels monitored until an acceptable antibody level (titer) had been reached.

For the preparation of monoclonal antibodies, one would follow standard techniques for the immunization of an animal, again using the decapeptide non-mammalian GnRH peptide or its analog. Once sufficiently high acceptable antibodies are reached (titer) in the animal, the spleen of the animal would be harvested and then fused with an immortalized cell line, such as a cancer cell line, to produce a population of hybridoma cells. This hybridoma population of cells would then be screened for those cells that produce the highest amount of antibody that specifically binds the non-mammalian GnRH analog decapeptide. Such hybridoma cells would be selected, and then cultured. The antibody to non-mammalian GnRH would then be collected from the media of the cell culture using techniques well known to those of skill in the art.

For purposes of the practice of preparing polyclonal and monoclonal antibody, the textbook Sambrook et al (1989) *Molecular Cloning, A Laboratory Manual,* @d Ed., Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y., is specifically incorporated herein by reference. In addition to the embodiments presented it is believed by Applicants that the disclosed non-mammalian GnRH isomers and/or analogs as well as any other gene regulator can be used to regulate the gene expression of non-mammalian GnRH or expression of its receptors in tumor cells. It is further believed by Applicants that the non-mammalian GnRH analogs disclosed can be used in the development of stable, toxin conjugated antibodies or ligands that can specifically bind to the GnRH receptor on the tumor cell and kill the

cell.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described therein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents, who are both chemically and physiologically, related, might be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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WE CLAIM:

1. A composition comprising a non-mammalian GnRH analog for use in regulating tumor GnRH activity, wherein said analog is capable of binding to tumor GnRH receptors with greater affinity than mammalian GnRH and is active in the presence of a post-proline peptidase or endopeptidase.

2. The composition of Claim 1 wherein said non-mammalian GnRH analog has a D-amino acid substitution at position 6 and a post-proline peptidase inhibitor at position 10.

3. The composition of Claim 2 wherein said post-proline peptidase inhibitor is selected from the group consisting of aza-Gly-amide or ethylamide.

4. The composition of Claim 2 wherein said non-mammalian GnRH analog is further defined as an anti-tumor agent.

5. The composition of Claim 2 wherein said non-mammalian GnRH analog is further defined as an anti-proliferative agent.

6. The composition of Claim 5 wherein said non-mammalian GnRH analog is further defined as an anti-metastatic agent.

7. The composition of Claim 6 wherein said non-mammalian GnRH analog is further defined as an apoptotic agent.

8. The composition of Claim 2 wherein said non-mammalian GnRH analog is selected from the group consisting of Chicken II GnRH analog, Salmon GnRH analog, and Herring GnRH analog.

9. The composition of Claim 8 wherein said Chicken II GnRH analog is selected from the group consisting of D-Arg(6)-Chicken II GnRH-ethylamide and D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide.

10. The composition of Claim 8 wherein said Salmon GnRH analog is selected from the group consisting of D-Arg(6)-Salmon-GnRH-ethylamide and D-Arg(6)-Salmon-GnRH-aza-Gly(10)-amide.

11. The composition of Claim 9 wherein said D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide has a sequence as defined in SEQ ID NO. 2.

12. The composition of Claim 8 wherein said Chicken II GnRH has a cDNA

sequence of SEQ ID NO. 1.

13. The composition of Claim 10 wherein said D-Arg(6)-Salmon-GnRH-aza-Gly(10)-amide has a sequence as defined in SEQ ID NO. 4.

14. The composition of Claim 8 wherein said Salmon GnRH has a cDNA sequence of SEQ ID NO. 3.

15. The composition of Claim 8 wherein said Herring GnRH analog has a sequence as defined in SEQ ID NO. 6.

16. The composition of Claim 8 wherein said Herring GnRH has a cDNA sequence of SEQ ID NO. 5.

17. The composition of Claim 2 wherein said D-amino acid substituted at position 6 is selected from a group consisting of D-Arg, D-Leu, D-tBu-Ser, and D-Trp.

18. A method for regulating tumor activity using a non-mammalian GnRH analog comprising the step of administering to a mammal a pharmaceutical preparation of the analog, wherein said analog is capable of binding to tumor GnRH receptors with greater affinity than mammalian GnRH and is active in the presence of a post-proline peptidase or endopeptidase due to a D-amino acid substitution at position 6 and a post-proline peptidase inhibitor at position 10.

19. The method of Claim 18 wherein said non-mammalian GnRH analog is selected from the group consisting of Chicken II GnRH analog, Salmon GnRH analog, and Herring GnRH analog.

20. The method of Claim 19 wherein said Chicken II GnRH analog is selected from the group consisting of D-Arg(6)-Chicken II GnRH-ethylamide and D-Arg(6)-Chicken II GnRH-aza-Gly(10)-amide.

21. The method of Claim 19 wherein said Salmon GnRH analog is selected from the group consisting of D-Arg(6)-Salmon-GnRH-ethylamide and D-Arg(6)-Salmon-GnRH-aza-Gly(10)-amide.

SEQ ID NO: 1
Chicken II
cDNA
CAG CAC TGG TCT CAT GGC TGG TAT CCT GGA

SEQ ID NO: 2
Chicken II GnRH Analog
p-Glu-His-Trp-Ser-His-D-Arg-Trp-Tyr-Pro-aza-Gly-NH_2

SEQ ID NO: 3
Salmon GnRH
cDNA
CAG CAC TGG TCT TAT GGC TGG CTG CCT GGA

SEQ ID NO: 4
Salmon GnRH Analog
p-Glu-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-Pro-aza-Gly-NH_2

SEQ ID NO: 5
Herring GnRH
cDNA
CAG CAC TGG TCT TAT GGC TGG CTG CCT GGA

SEQ ID NO: 6
Herring GnRH Analog
p-Glu-His-Trp-Ser-Tyr-D-Arg-Leu-Ser-Pro-aza-Gly-NH_2

1	2	3	4	5	6	7	8	9	10	
p-Glu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-NH_2	Mammalian
p-Glu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Gln-	Pro-	Gly-NH_2	Chicken I
p-Glu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly-NH_2	Salmon
p-Glu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Tyr-	Pro-	Gly-NH_2	Chicken II
p-Glu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Leu-	Pro-	Gly-NH_2	Dogfish
p-Glu-	His-	Trp-	Ser-	His-	Gly-	Leu-	Ser-	Pro-	Gly-NH_2	Herring
p-Glu-	His-	Trp-	Ser-	His-	Gly-	Leu-	Asn-	Pro-	Gly-NH_2	Catfish
p-Glu-	His-	Trp-	Ser-	His-	Asp-	Trp-	Lys-	Pro-	Gly-NH_2	Lamprey III
p-Glu-	His-	Tyr-	Ser-	Leu-	Glu-	Trp-	Lys-	Pro-	Gly-NH_2	Lamprey I

Fig. 1

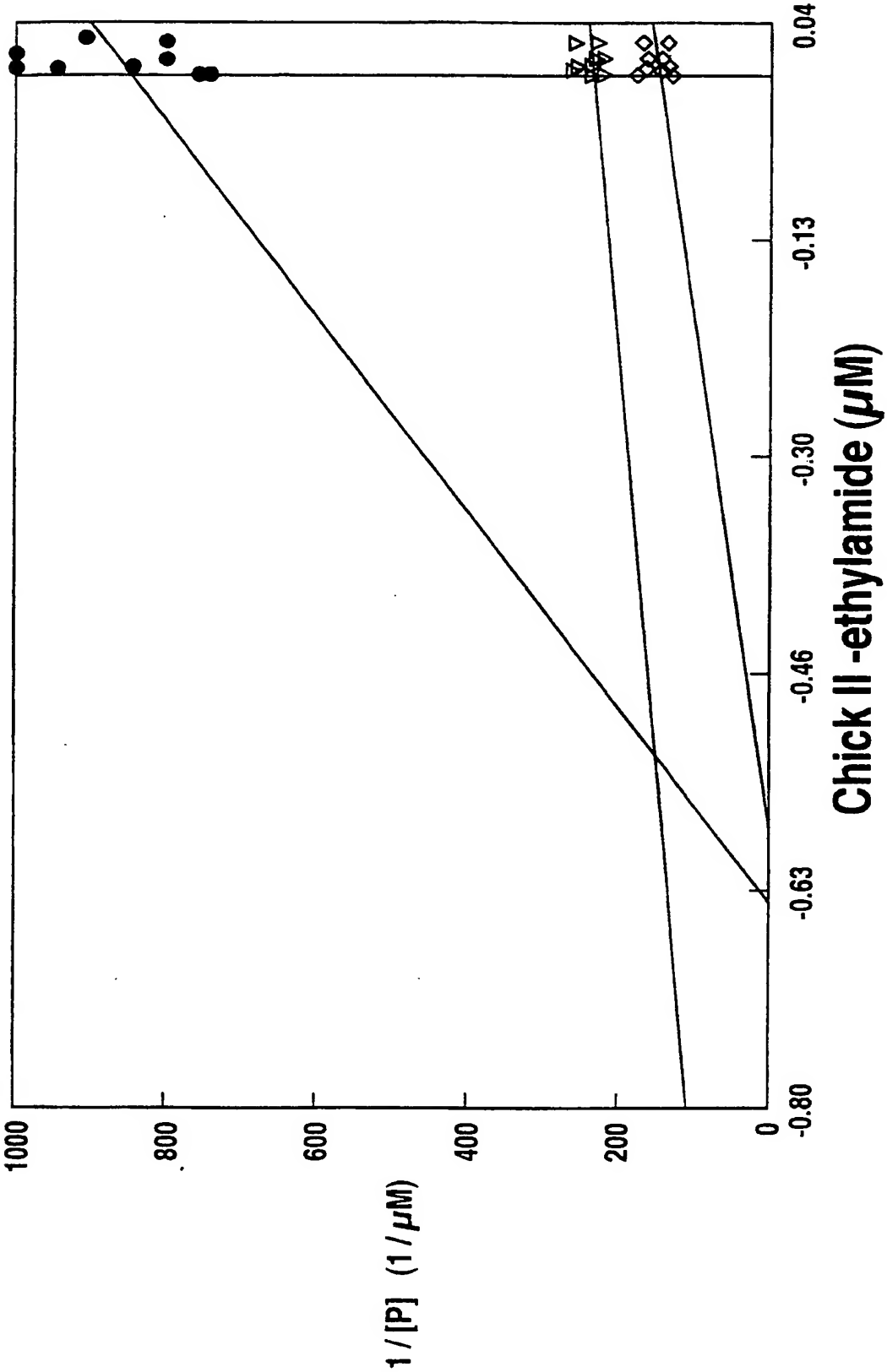
1000
800
600
400
200
0
1 / [P] (1 / μM)
0.04
-0.13
-0.30
-0.46
-0.63
-0.80
Chick II -ethylamide (μM)

Fig. 2

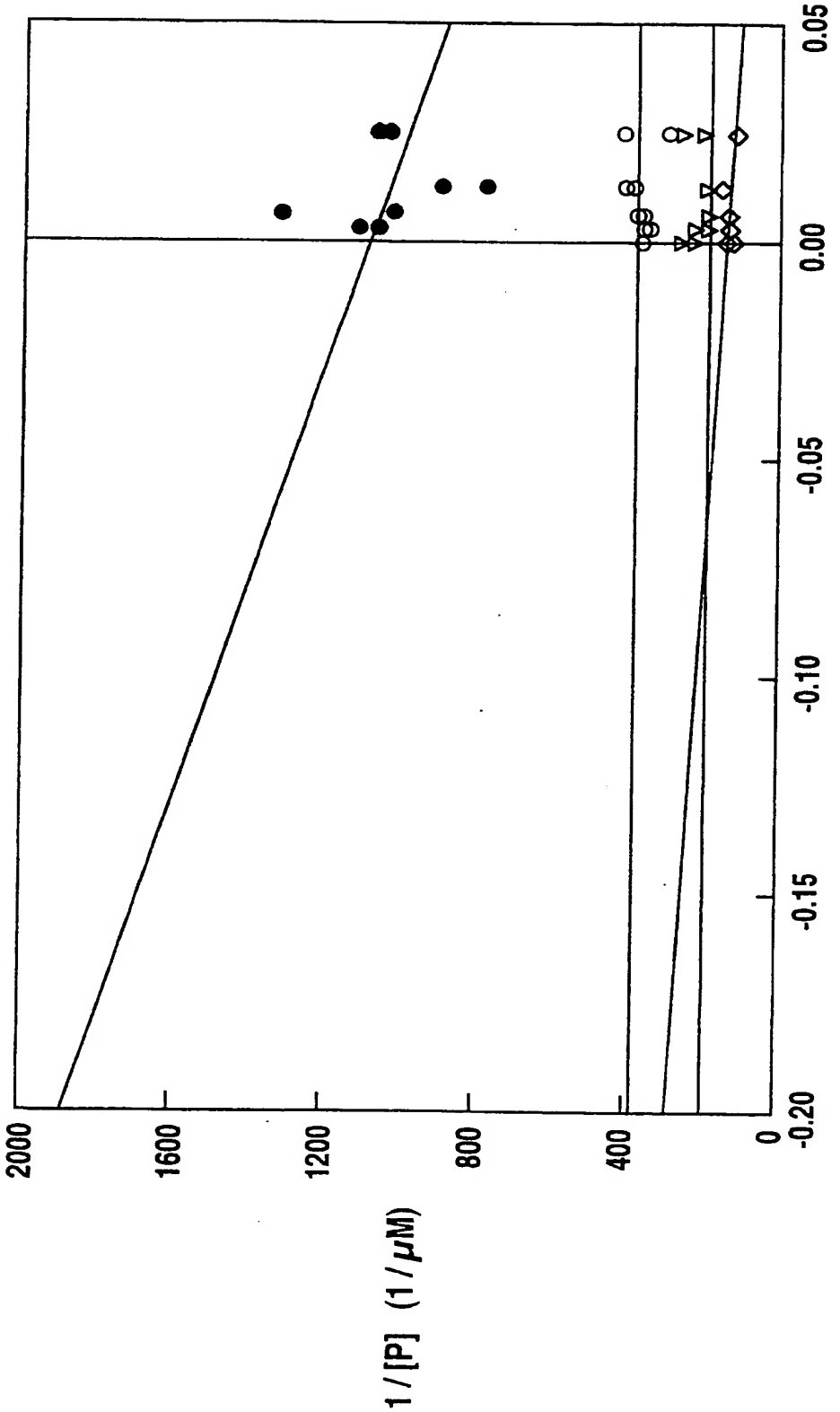
2000
1600
1200
800
400
0
1 / [P] (1 / μM)
0.05
0.00
-0.05
-0.10
-0.15
-0.20
D-Arg(6)-Chicken II-aza-Gly(10)-amide (μM)

Fig. 3

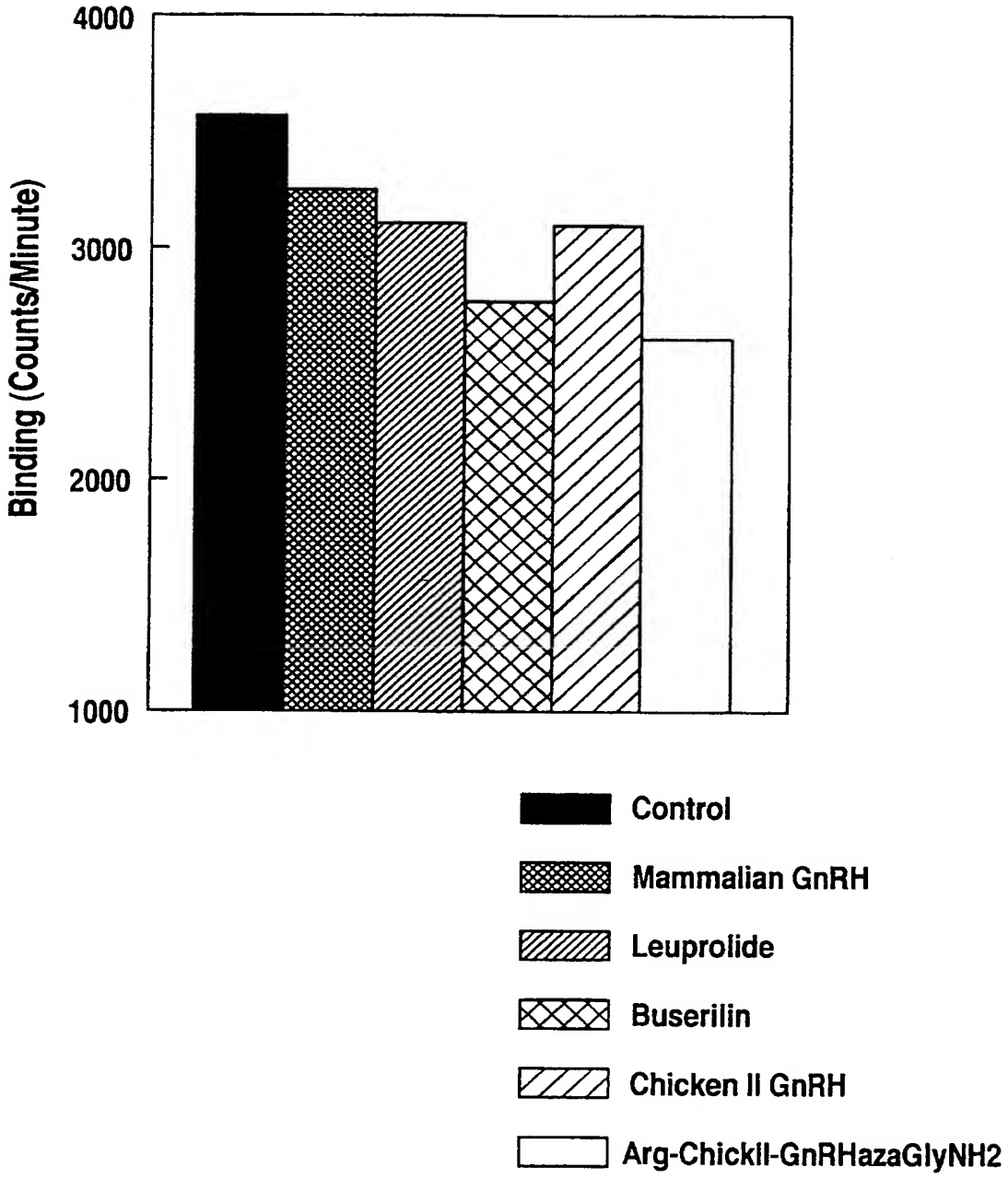
4000
3000
2000
1000
Binding (Counts/Minute)
Control
Mammalian GnRH
Leuprolide
Buserilin
Chicken II GnRH
Arg-ChickII-GnRHazaGlyNH2

Fig. 4

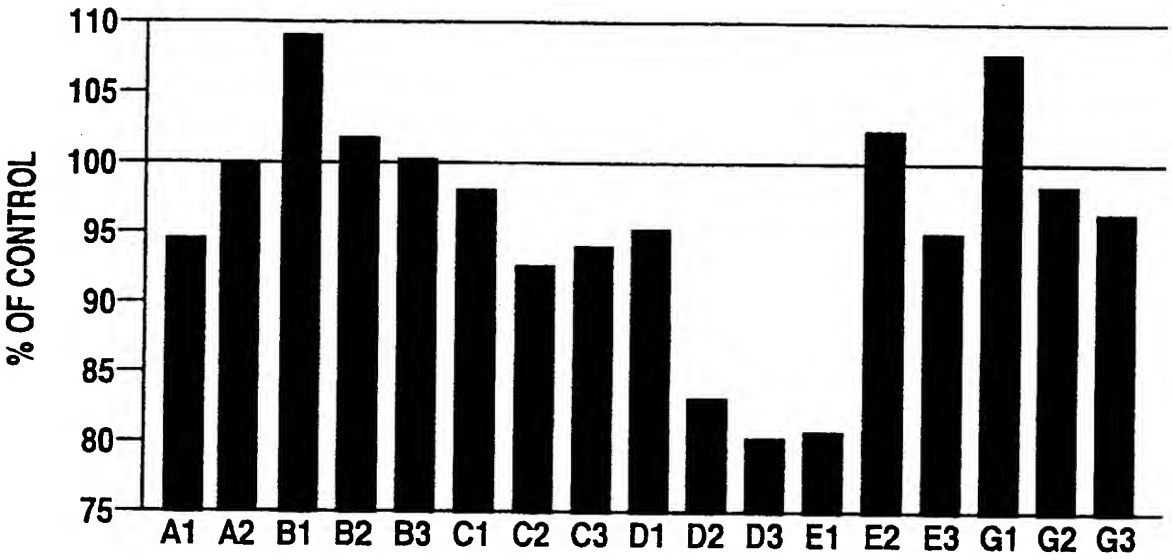
% OF CONTROL
110
105
100
95
90
85
80
75
A1
A2
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
G1
G2
G3

Fig. 5

Analog of GnRH	
Mammalian	30
Lamprey	20
Salmon	300
Chicken I	80
Chicken II	200
Chicken II EA (10)	130
Chicken II D-Arg (6), aza-Gly (10) amide	>200
Salmon II D-Arg (6), aza-Gly (10) amide	200
Mammalian D-Trp (6)	20
Mammalian EA (10)	70
Mammalian D-Trp (6), EA (10)	60
Mammalian D-Leu (6), EA (10)	80
Mammalian But-D-Ser (6), EA (10)	110
Mammalian Im-bzl-D-His (6), EA (10)	>200
Antide	120

Fig. 6

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 00/26575

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/09 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NICHOLSON R I ET AL: "ANTITUMOR ACTIVITY OF FOLLIGEN A NOVEL GONADOTROPIN-RELEASING HORMONE ANALOGUE AGAINST DMBA-INDUCED TUMOURS IN THE RAT" TUMOR BIOLOGY, vol. 13, no. 1-2, 1992, pages 44-50, XP001002047 ISSN: 1010-4283	1-8, 17-19
Y	the whole document	9-16,20, 21
Y	GB 2 237 571 A (MILLAR ROBERT PETER) 8 May 1991 (1991-05-08) abstract page 1 -page 3, paragraph 7 page 6, paragraph 2 - paragraph 3	9-16,20, 21

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2001	29/05/2001

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KARTEN M J ET AL: "GONADOTROPIN-RELEASING HORMONE ANALOG DESIGN. STRUCTURE-FUNCTION STUDIES TOWARD THE DEVELOPMENT OF AGONISTS AND ANTAGONISTS: RATIONALE AND PERSPECTIVE" ENDOCRINE REVIEWS,US,BALTIMORE, MD, vol. 7, no. 1, 1986, pages 44-66, XP002038872 the whole document	1-21
X	US 5 760 000 A (HABIBI HAMID R) 2 June 1998 (1998-06-02) abstract column 2, line 10 - line 24 column 3, line 16 - line 25 column 5, line 9 - line 52 examples 2,8-10	1-5,17, 18

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatl Application No
PCT/US 00/26575

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2237571 A	08-05-1991	CA 2029018 A	02-05-1991
		ZA 9008738 A	28-08-1991
US 5760000 A	02-06-1998	AU 2265895 A	05-12-1995
		WO 9531475 A	23-11-1995